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CHEMICAL ABSTRACTS, vol. 92, no. 14, 7th April 1980, page 329, column 1, abstract no. 116045x, Columbus, Ohio, US; Y. MASAO "Application of a marine methanol-utilizing bacterium for bioassay of vitamin B12 in sea water", & **FERMENT. TECHNOL.** 1979, 57(5), 400-407

PATENT ABSTRACTS OF JAPAN, vol. 4, no. 46 (C-6)[528], 10th April 1980; & JP - A - 55 19022 (SANRAKU OCEAN K.K.) 09-02-1980 (Cat. D)

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DescriptionField of the Invention

5 The present invention relates to a method of quantitative microorganism assay for a vitamin B₁₂ - (hereafter simply referred to as B₁₂)-active substance and a reagent used for the method of quantitative assay. More particularly, the present invention relates to a method of quantitative assay for the B₁₂-active substance using marine methanol-utilizing bacteria requiring B₁₂ for growth and capable of assimilating methanol quickly in a simple manner and a reagent for quantitative assay of the B₁₂-active substance
 10 comprising a composition of dry viable bacteria for inoculation of marine methanol-utilizing bacteria having B₁₂ auxotrophy used for the quantitative assay and a liquid medium containing methanol and also containing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester and a B₁₂ standard solution.

Brief Description of the Prior Art

Physicochemical methods and microbiological methods have been hitherto known as quantitative assay for B₁₂-active substances. Among the microbiological methods, a method of quantitatively determining B₁₂-active substances using marine methanol-utilizing bacteria requiring B₁₂ for growth is known to be excellent
 20 one (Published Unexamined Japanese Patent Application No. 19022/1980).

In case that an attempt is made to quantitatively determine B₁₂-active substances, however, this method is disadvantageous that bacteria do not necessarily have stable growth and the B₁₂-active substances cannot be quantitatively determined quickly with accuracy.

Problems to be solved by the present invention are in the development of an improved method for
 25 quantitatively assaying the B₁₂-active substance that eliminates the defects possessed by conventional assay methods of the B₁₂-active substances using marine methanol-utilizing bacteria having B₁₂ auxotrophy, namely, failing to quantitatively determine the B₁₂-active substance stably and quickly with good accuracy, and reagents for quantitative assay for B₁₂.

As a result of various investigations to solve the foregoing problems, the present inventors have found
 30 that by supplementing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester to a medium for quantitative assay using marine methanol-utilizing bacteria having B₁₂ auxotrophy, the B₁₂-active substance can be quantitatively determined stably with accuracy. The present invention has been accomplished based on this finding.

Summary of the Invention

An object of the present invention is to provide a quick and simple method of quantitative assay for the B₁₂-active substances using marine methanol-utilizing bacteria.

Another object of the present invention is to provide a reagent used in the quantitative assay for the
 40 B₁₂-active substances using marine methanol-utilizing bacteria.

The method of quantitative assay for a vitamin B₁₂-active substance according to the present invention is characterized by culturing marine methanol-utilizing bacteria having B₁₂ auxotrophy in a medium for quantitative assay for the bacteria containing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester and, quantitatively determining vitamin B₁₂ depending upon degree of growth thereof.

45 The present invention is also directed to a reagent for quantitative assay used in microbiological assay for the B₁₂ -active substance, comprising a kit for combination of an ampoule or vial having sealed therein a dry viable bacteria composition of marine methanol-utilizing bacteria having B₁₂ auxotrophy for inoculation and an ampoule or vial having sealed therein a medium for quantitative assay for the bacteria containing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester, or further an ampoule or vial
 50 having sealed therein a standard dilution of vitamin B₁₂ having a serial concentration.

Detailed Description of the Invention

As microorganisms which can be used in accordance with the present invention, mention may be made
 55 of *Alteromonas thalassomethanolica* YK 4007, FERM BP-1401 (This strain deposited under FERM P-3621 on June 22, 1976 at Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi, I-chome, Yatabe-machi, Tsukuba-gun, Ibaraki-ken, Japan and Transferred on July 2, 1987 to International deposition FERM BP-1401 under the treaty on the International Recognition of the Deposit of

Microorganisms for the purpose of patent procedure. Its bacteriological properties are described in J. Ferment. Technol., 58, 99-106 (1980)) which has already been separated as a marine methanol-utilizing bacteria based on characteristics that the bacteria can be well assimilated using methanol as only one carbon source and seawater can be utilized as water for the medium, etc.; and the like. All of these microorganisms are characterized by requiring B₁₂ for growth.

As media for quantitatively assaying the B₁₂-active substance that can be used in the present invention, conventional media for quantitatively assaying marine methanol-utilizing bacteria having B₁₂ auxotrophy heretofore used are usable except for that containing pyrroloquinoline-quinone and the polyoxyethylene sorbitan fatty acid ester. Additional incorporation of iron sulfates, zinc sulfates, sodium thiosulfate, thioglycolic acid, etc. in the media having the aforesaid composition for quantitative assay give good assay results in some occasion.

It is desired that the amount of pyrroloquinoline-quinone to be incorporated is in the range of 10 to 60 ng per 100 ml of the medium for quantitative assay; when the amount exceeds 75 ng, good assay results cannot always be obtained because the growth of the microorganism is inhibited. Further when the amount is less than 10 ng, good assay results cannot always be obtained because of uneven growth of the microorganism.

preferably the amount of the polyoxyethylene sorbitan fatty acid ester to be incorporated should be in the range of 1 to 20 mg based on 100 ml of the medium for quantitative assay; when the amount exceeds 20 mg, the growth of the microorganism is sometimes inhibited and when the amount is less than 1 mg, uneven growth is noted and good results cannot always be obtained.

The inoculant that can be used in the present invention may be used by preincubating the bacteria used in the liquid medium, collecting the bacteria and washing, as in conventional microbiological methods; the inoculant may also be used without preincubation, by collecting the bacteria grown on agar in a conventional manner and washing. It is also a characteristic of the present invention that the quantitative assay can be performed in a simpler manner.

In the quantitative assay method of the present invention, no growth of bacteria is observed with a variety of substances that lack B₁₂ activity for example, yeast extract that contains DL-methionine which is an amino acid, thymidine which is one of nucleic acid components, δ -aminolevulinic acid which is a precursor of biosynthesis, cobalt salts which are constituents of B₁₂, various amino acids, peptides, vitamins and nucleic acids but does not contain B₁₂, etc.; growth of the bacteria is observed with meat extract containing B₁₂. From such it is clear that the B₁₂-active substance can be specifically determined quantitatively according to the assay method of the present invention.

The microorganism used in the present invention can be dried while maintaining a live state and, the composition of the dry bacteria can be stored over a long period of time while the microorganism is in a live state. Accordingly, when the viable bacteria are dried and stored, the dry composition can be readily suspended in a medium solution or sterilized water upon quantitative assay for the B₁₂-active substance and the bacteria suspension can be used directly as a inoculated bacteria solution for quantitative assay. Further, the liquid culture solution for quantitative assay can be aseptically stored over a long period of time, sealed in, for example, an ampoule, a glass bottle, etc., stored, unsealed depending upon necessity and, provided for assay as a set or kit together with the composition of the dry bacteria for inoculation described above. For this reason, the composition of the bacteria and a predetermined amount of the medium for quantitative assay comprising pyrroloquinoline-quinone and the polyoxyethylene sorbitan fatty acid ester can be provided as an assay device; when assaying the B₁₂-active substance quantitatively using the reagent, it can be accomplished in an extremely simple manner without requiring storage and control of the bacterial strain. Further, an automated bioassay device can be constructed by incorporating the bacteria composition and the medium for quantitative assay in accordance with the present invention into the device, respectively to quantitatively assay the B₁₂-active substance automatically. To dry the viable bacteria, a solution of 10 g of sodium chloride, 20 g of sodium glutamate and 0.2 g of magnesium sulfate in 1 liter of M/30 phosphate buffer is used as a dispersion medium and the washed viable bacteria is suspended in the dispersion medium followed by freeze drying. It is advantageous that the medium for quantitative assay be stored and supplied as an ampoule because such is free from evaporation and dissipation of methanol. The bacteria are resistant to salts, grow using methanol as only one carbon source and mostly insensitive to components other than B₁₂ so that even extremely rough operation can give accurate analytical data.

Next the present invention will be described by referring to the examples.

Example 1

A medium for storing the bacterial strain was prepared as follows. To 1 liter of water were added 2 g of

ammonium sulfate, 0.3 g of magnesium sulfate, 1 g of potassium primary phosphate, 5.5 g of potassium secondary phosphate, 30 g of sodium chloride, 0.5 g of meat extract and 15 g Of agar. After Sterilizing at 1200 ° C for 5 minutes, 5 ml of methanol was added before agar was solidified. The system was separately poured in sterilized test tubes, which were slanted and solidified to make a slant, respectively. An assay medium was prepared by sterilizing 1 liter of a medium for quantitative assay having a composition shown in Table 1 at 120 ° C for 5 minutes, adding 2.5 ml of methanol thereto and separately pouring the medium for quantitative assay into an L-shaped tube by 9 ml each.

Table 1

Component	Medium A (/l)	Medium B (/l)
Ammonium sulfate	1 g	1 g
Magnesium sulfate	0.3g	0.3g
Potassium primary phosphate	2 g	2 g
Potassium secondary phosphate	7 g	7 g
Sodium chloride	30 g	30 g
Pyrroloquinolinequinone	-	300 ng
"Tween 80"	-	100 mg

As samples for analysis, "ERENTAL" or parenteral nutrient manufactured by Ajinomoto Co., Inc., sardine meat and mackerel meat were used. 10 samples of 1 g each were taken and immersed in 10 ml of deionized water, respectively. After homogenization, heat treatment was conducted at 100 ° C for 30 minutes in a hot water bath. The treated liquids were centrifuged and each supernatant was appropriately diluted. The dilutions were made test sample solutions.

Alteromonas thalassomethanolica YK 4007 FERM P-1401 was inoculated on the slant described above. After incubation at 30 ° C for a day, the system was stored at 5 ° C. Upon use, bacteria grown on the slant was suspended in Medium A and Medium B described above which contained methanol. After once rinsing with the same medium, respectively, dilution was performed in the same medium solution in such a manner that absorbancy (OD) became 0.3 at 610 nm. Thus, bacteria solutions for inoculation were obtained. A standard cyanocobalamin solution and test sample solutions were charged by 1 ml each into L-shaped tubes charged with 9 ml each of the aforesaid Medium A and Medium B containing methanol. Further 0.2 ml of the aforesaid bacteria solution for inoculation was added thereto followed by shake culture at 35 ° C for 15 hours. The absorbancy of the culture solution was measured as an OD value at 610 nm to prepare a standard curve, based on which the amount of cyanocobalamin corresponding to the OD value of the test sample was determined. The results are shown in Table 2.

Table 2

Sample	Methanol-Containing Medium A		Methanol-Containing Medium B	
	Mean Value of 10 Samples (ng/g)	Maximum Value (ng/g)	Mean Value of 10 Samples (ng/g)	Maximum Value (ng/g)
ERENTAL	8.5	9.0	8.7	8.9
Sardine meat	55.3	59.8	53.8	54.3
Mackerel meat	10.2	10.9	9.8	10.1

Example 2

The amount of cyanocobalamin contained in "ERENTAL" was quantitatively assayed in a manner similar to Example 1 except that a medium shown in Table 3 was used as a basal medium, a medium for

quantitative assay supplemented with pyrroloquinoline-quinone having a concentration shown in Table 4 and "Tween 80" was used and "ERENTAL" or parenteral nutrient as used in Example 1 was used.

Table 3

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Medium for quantitative assay:

KH₂PO₄ 1.0 g

K₂HPO₄ 3.0 g

(NH₄)₂SO₄ 1.0 g

10 MgSO₄·7H₂O 0.3 g

NaCl 30.0 g

NaS₂O₃·5H₂O 10.0 mg

ZnSO₄·7H₂O 30.0 μg

FeSO₄·7H₂O 5.0 mg

15 Methanol 5.0 ml

Water 1000 ml

pH 6.8

The results are shown in Table 4.

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Table 4

Addition Concentration

Pyrrolo- quinoline- quinone (ng/100 ml)	Tween 80 (ng/100 ml)	Mean Value of 10 Samples (ng/100 ml)	Maximum Value (ng/100 ml)	Minimum Value (ng/100 ml)
0	0	8.5	9.0	8.0
8	0	8.5	8.9	8.0
10	1	8.5	8.6	8.4
30	10	8.5	8.6	8.4
60	20	8.5	8.6	8.4
75	20	6.2	6.4	6.0

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Claims

1. A method of quantitative assay for a vitamin B₁₂-active substance which comprises culturing marine methanol-utilizing bacteria having Vitamin-B₁₂ auxotrophy in a medium for quantitative assay of said Vitamin-B₁₂-active substance said medium containing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester and, quantitatively determining the vitamin-B₁₂ active substance by the degree of growth of said bacteria

2. A kit for the quantitative microbiological assay for vitamin B₁₂-active substances as claimed in claim 1 which comprises an ampoule or vial having sealed therein a dry viable bacteria composition of marine methanol-utilizing bacteria having vitamin-B₁₂ auxotrophy for inoculation and an ampoule or vial having sealed therein a medium for quantitative assay of said bacteria containing pyrroloquinoline-quinone and a polyoxyethylene sorbitan fatty acid ester, and further an ampoule or vial having sealed therein a standard dilution of vitamin-B₁₂.

Revendications

1. Procédé d'essai quantitatif d'une substance agissant comme la vitamine B₁₂, qui comprend les opérations consistant à cultiver une bactérie utilisant du méthanol marin et à auxotrophie en vitamine B₁₂, dans un milieu pour un essai quantitatif de ladite substance agissant comme la vitamine B₁₂, ledit milieu contenant de la pyrroloquinoline-quinone et un ester d'acide gras de polyoxyéthylènesorbitanne, et à déterminer quantitativement la substance active agissant comme la vitamine B₁₂, par le degré de croissance de ladite bactérie.
2. Kit pour l'essai quantitatif microbiologique de substances agissant comme la vitamine B₁₂ selon la revendication 1, qui comprend une ampoule ou un flacon fermé(e) hermétiquement et contenant une composition bactérienne sèche et viable constituée d'une bactérie utilisant du méthanol marin et à auxotrophie en vitamine B₁₂, destinée à être inoculée, et une ampoule ou un flacon fermé(e) hermétiquement et contenant un milieu pour l'essai quantitatif de ladite bactérie, contenant de la pyrroloquinoline-quinone et un ester d'acide gras de polyoxyéthylènesorbitanne, et aussi une ampoule ou un flacon fermé(e) hermétiquement et contenant une dilution type de vitamine B₁₂.

Patentansprüche

1. Verfahren zur quantitativen Untersuchung auf eine Vitamin-B₁₂-aktive Substanz, das umfaßt:
- Kultivierung von marinen Methanol verwertenden Bakterien, die Vitamin-B₁₂ Auxotrophie in einem Medium zur quantitativen Untersuchung dieser Vitamin-B₁₂-aktiven Substanz aufweisen, wobei dieses Medium Pyrrolochinolin-chinon und einen Polyoxyethylensorbitanfettsäureester enthält und,
 - quantitative Bestimmung der Vitamin-B₁₂-aktiven Substanz mittels des Wachstumsgrades dieser Bakterien.
2. Kit für die quantitative mikrobiologische Untersuchung auf Vitamin-B₁₂-aktive Substanzen gemäß Anspruch 1, der umfaßt:
- eine Ampulle oder ein Glasfläschchen, die (das) verschlossen darin ein Medium zur quantitativen Untersuchung dieser Bakterien aufweist, das Pyrrolochinonin-chinon und ein Polyoxyethylensorbitanfettsäureester enthält, und weiter
 - eine Ampulle oder ein Glasfläschchen, die (das) verschlossen darin eine Standard-Vitamin-B₁₂-Verdünnung enthält.